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Cell-Mediated Immune Responses to *Babesia bovis* Merozoite Antigens in Cattle following Infection with Tick-Derived or Cultured Parasites

WENDY C. BROWN,* KATHLEEN S. LOGAN, GERALD G. WAGNER, AND CHRISTINE L. TETZLAFF

Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas 77843

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Peripheral blood mononuclear cells from cattle experimentally infected with *Babesia bovis* were examined for parasite-specific cell-mediated immune responses. Unfractionated merozoites and soluble and membrane fractions derived from merozoites were all antigenic for immune cattle, although the membrane fraction was the most stimulatory. Cattle responded to different antigenic fractions in a differential manner, and only that animal immunized with autologous cultured parasites responded to parasitized erythrocyte culture supernatants. Plastic-adherent cells (presumably monocytes/macrophages) were required for a proliferative response to babesial antigens but not to the T-cell mitogen concanavalin A, suggesting that babesial proteins are not simply mitogenic for T cells. Lymphocyte responses directed against a different hemoparasite from Mexico, *Babesia bigemina*, indicate that this parasite shares cross-reactive T-cell epitopes with *B. bovis*. These studies define a system whereby T lymphocytes from babesia-immune cattle can be used in proliferation assays to identify babesial merozoite antigens which are immunogenic for T cells. Because identification of helper T-cell epitopes is important for the design of a babesial subunit vaccine which will evoke anamnestic responses, the studies described here provide a basis for such experiments.

Babesiosis is a major hemoparasitic disease of cattle in semitropical regions of the world which results in severe economic losses to the livestock industry. Transmitted by the ixodid tick *Boophilus microplus*, *Babesia bovis* infections in susceptible cattle are characterized by fever, anorexia and cachexia, low parasitemia, and a generalized circulatory disturbance which includes sequestration of parasitized erythrocytes in the capillary beds of the brain and lung. Death is often caused by a respiratory distress syndrome associated with massive infiltration of neutrophils and parasitized erythrocytes into the lung capillaries, resulting in vascular permeability and edema (47, 48). Cytokines released by parasite-activated T cells and monocytes are most likely involved in the pathophysiology of this disease (47, 48), as has been demonstrated with plasmodium-infected mice (7, 14, 27).

Animals that survive natural infection, or recover from experimental infection with attenuated parasites, are protected against virulent challenge (3), and partial immunity against homologous and heterologous challenge can be induced by vaccination with crude parasite extracts (24, 25) and soluble culture-derived exoantigens (18, 28, 39, 42). However, vaccination with living parasites poses a number of problems, not the least of which is establishment of a carrier state which permits continual babesial transmission. These observations provide the basis for developing a nonlive vaccine for babesiosis.

Although antibody is important in controlling babesial infection (23), antibody alone cannot account for the patterns of immunity observed in response to infection and challenge with heterologous *B. bovis* parasites (22) and does not always correlate with protection (41). In addition to providing B-cell help, T cells are the primary effector cells in protective immunity against many protozoal infections (9, 17, 29, 36, 37, 38), including *Babesia microti* infections (33).

Few published studies have examined the role of T lymphocytes in immunity to *Babesia bovis*. Timms et al. (43) reported that peripheral blood mononuclear cells (PBMC) from cattle protectively immunized with attenuated live parasites exhibited weak and short-lived proliferative responses to a crude soluble parasite extract. Animals immunized with exoantigen had stronger cell-mediated responses but were less protected upon challenge. However, in both groups of cattle, lymphocyte proliferation and antibody responses correlated with protection when cattle were challenged at different times after vaccination. As a strategy for developing vaccines which would include T-cell epitopes important in the induction of a protective immune response (6), we have focused our research on characterizing T-cell-immunodominant antigens and T-cell responses in cattle protectively immunized against *B. bovis*. In the studies reported here, we have examined proliferative responses of PBMC against crude membrane and soluble parasite extracts and soluble exoantigen from cattle immunized with virulent tick-derived parasites or avirulent cultured parasites. Vigorous proliferation was observed in response to crude parasite antigens for at least 2 years following challenge (as of this writing). These experiments provide the basis for an in-depth functional analysis of defined populations of T cells and monocytes from these and other babesia-immune cattle in response to defined babesial antigens.

MATERIALS AND METHODS

Babesial parasite strains and in vitro cultivation. The *B. bovis* parasites used for in vivo infection and challenge studies originated from a Mexican isolate, designated isolate 222, obtained from naturally infected cattle in a babesia-endemic area of Mexico in 1978. Parasites were maintained in a tick colony at the U.S. Department of Agriculture

* Corresponding author.

laboratory in Mission, Tex. In vitro erythrocyte cultures derived from this isolate of *B. bovis* were maintained as described by Holman et al. (16), using a modification of the microaerophilus stationary-phase (MASP) culture system (20). In vitro cultures of *Babesia bigemina* parasites were maintained under the same culture conditions. The *B. bigemina* parasites were obtained in 1982 from a naturally infected animal in northeastern Mexico. Parasites were passaged four times in splenectomized calves by subinoculation of blood containing parasitized erythrocytes, and washed, packed erythrocytes obtained during peak parasitemia were mixed with an equal volume of phosphate-buffered saline (PBS) containing 4 M dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), aliquoted, and cryopreserved in liquid nitrogen. This parasite stock, or stabilate (21), was then used to initiate the parasite cultures.

Experimental cattle. Animal C15, a spleen-intact, 2-year-old European crossbred cow, was inoculated intravenously two times, 1 month apart, with 2×10^6 and 4×10^6 erythrocytes derived from MASP cultures of autologous, *B. bovis*-infected erythrocytes. Three years later, the cow was inoculated again with 7×10^9 cultured parasitized erythrocytes, and PBMC were monitored thereafter for responses to parasite antigens. Animals C97 and C88, 9-month-old spleen-intact Brahman-European crossbred cows, were infected with the Mexican isolate of *B. bovis* by infestation with *B. bovis*-infected *Boophilus microplus* tick larvae (1 g; approximately 20,000). Both animals experienced clinical babesiosis and were treated by intramuscular inoculation of diminazene aceturate (Ganaseg; 3 mg/kg; Squibb and Sons, Mexico City, Mexico) diluted in H_2O. Three months later, the animals were challenged by intramuscular inoculation of 2.5 ml of ground-up tick stabilate (GUTS) prepared from *B. bovis*-infected ticks collected from animal C97 on day 3 postinfection. GUTS was prepared in the following way. Engorged ticks (2 g; approximately 10,000) were homogenized with a loose-fitting Ten-Broeck tissue homogenizer in 15 ml of RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 3.5% bovine serum albumin. The homogenate was centrifuged for 5 min at $250 \times g$, and the supernatant was collected, layered over 3 ml of Ficoll-Hypaque (Histopaque-1077; Sigma), and centrifuged for 5 min at $150 \times g$. The supernatant above the Ficoll-Hypaque interface was collected, diluted in an equal volume of RPMI 1640 medium containing 4 M dimethyl sulfoxide, aliquoted, and rapidly frozen in liquid nitrogen. Animal C110, a spleen-intact, 9-month-old steer, served as a nonimmune control to assess the virulence of the GUTS challenge. Age-matched animal C99, a Brahman-European crossbred steer, served as a noninfected control for all experiments. All cattle were monitored daily for at least 2 weeks after infection for signs of infection by determining rectal temperature, packed erythrocyte volume (PCV), and peripheral parasitemia in Giemsa-stained thin blood films.

Parasite antigens. Parasites were cultured at 37°C in 5% CO_2 in air in 24-well plates (1.25 ml; Costar, Cambridge, Mass.) or in 25-cm^2 flasks (12.5 ml; Costar) containing a 5% (vol/vol) concentration of erythrocytes from animal C15 in medium 199 (GIBCO) supplemented with 40% autologous bovine serum. Merozoites were harvested following CO_2 deprivation (46) of cultures after the relative percentage of parasitized erythrocytes was increased by sequential reduction of the concentration of erythrocytes (13), with the following modifications. Infected erythrocyte cultures containing at least 15% parasitized erythrocytes were incubated for 18 h in serum-free HL-1 medium (Ventrex Laboratories, Inc., Portland, Maine) in 100% medical-grade oxygen at 37°C. Merozoites were recovered from the culture supernatant following a series of 10-min (140, 140, 180, and $220 \times g$) and 15-min ($5,000 \times g$) centrifugations of the supernatant, followed by two 15-min ($6,500 \times g$) centrifugations of the parasite pellet resuspended in PBS. Merozoites prepared in this manner contained some erythrocyte membranes but were relatively pure. Culture supernatants collected from *B. bovis* MASP cultures contain soluble parasite antigens designated exoantigens (32). The culture supernatant was collected after the $5,000 \times g$ centrifugation and is designated ISUP. Supernatants prepared in the same way from autologous, uninfected cultured erythrocytes are designated USUP. Merozoites (designated MER) were resuspended in PBS containing the protease inhibitors E-64 and Antipain (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 25 μg/ml. To prepare subcellular fractions, merozoites collected from 12 25-cm^2 flasks were disrupted by two passages through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) under a chamber pressure of 1,500 lb/in^2, and the homogenate was centrifuged for 1 h at $145,000 \times g$. The soluble high-speed supernatant fraction (designated HSS fraction) and membrane pellet resuspended in an equal volume of PBS (designated CM fraction) were collected. Fractions prepared in this manner generally yielded approximately 6 mg of protein in the CM fraction and 3 mg of protein in the HSS fraction. Ghosts prepared from uninfected erythrocytes (referred to as URBC) and a CM and HSS fraction derived therefrom were also prepared. Protein concentrations in the parasite or erythrocyte fractions were determined as described previously (2), using bovine immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) as a protein standard. All fractions were stored at −80°C in aliquots.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (19), using a Bio-Rad minigel apparatus, 10% acrylamide gels, and 10 μg of protein (consisting of babesial or uninfected erythrocyte antigens) per lane. Immunoblotting onto nitrocellulose was performed as described previously (44) for 1 h at 100 V, using the Bio-Rad Transblot apparatus. Blots were reacted with sera from normal cattle (C99 or preinfection sera) or *B. bovis*-immune cattle diluted 1:100 in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) containing 1% gelatin, and serologically reactive proteins were detected with a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-bovine immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as instructed by the manufacturers.

Lymphocyte proliferation assays. PBMC were prepared by Ficoll-Hypaque density gradient centrifugation of blood diluted 1:1 in Alsever's solution (10). PBMC were tested for reactivity to parasite antigens in a proliferation assay performed for 5, 6, or 7 days. However, in one experiment, lymphoproliferation was compared in duplicate assays harvested after 3 or 6 days. In another experiment, PBMC (2×10^6 cells per ml in a 25-cm^2 flask at 37°C) were allowed to adhere for 2 h to polystyrene, and the nonadherent fraction of the cells was compared with unseparated PBMC for lymphoproliferative responses. For all experiments, cells were cultured in complete medium, which consisted of RPMI 1640 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; GIBCO) and supplemented with 10% heat-inactivated fetal bovine serum

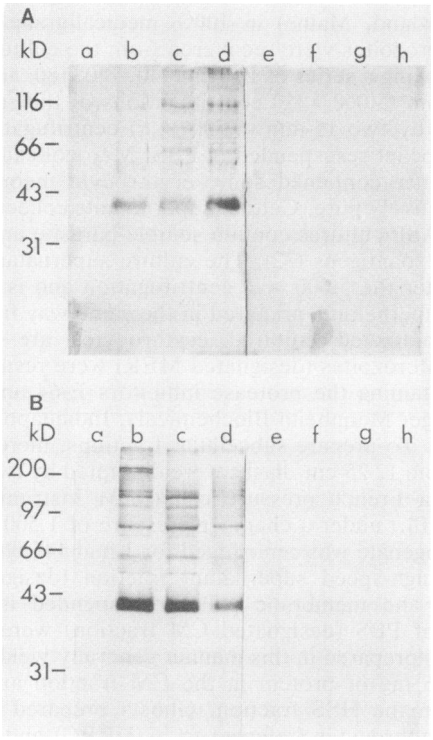

FIG. 1. Serologic reactivity of *B. bovis*-immune sera with babesial antigens. Western blots were performed by using preimmune (lanes e to h) or postchallenge (lanes a to d) sera from animal C15 (A) or C97 (B). (A) Lanes: a and h, URBC; b and g, parasite HSS; c and f, parasite CM; d and e, MER. (B) Lanes: a and h, URBC; b and g, MER; c and f, parasite CM; d and e, parasite HSS. Molecular size standards are indicated on the left.

(Hyclone, Logan, Utah), 2 mM L-glutamine (GIBCO), 5×10^{-5} M 2-mercaptoethanol (Sigma), and 50 μg of gentamicin sulfate (GIBCO) per ml. Proliferation assays were performed in 96-well flat-bottom plates (200 μl per well) or half-area plates (100 μl per well; Costar) with a final concentration of 2×10^6 PMBC per ml of complete medium. Concanavalin A (ConA; Sigma) or antigen (*B. bovis* MER, CM, and HSS; *B. bigemina* MER, URBC, and URBC CM) were added to the assays at final concentrations of 0.2 to 25 μg of protein per ml of complete medium. Exoantigens (ISUP or USUP) were added at a final concentration of 50 or 250 μg/ml. When present, indomethacin (Sigma) was added to the assays at a final concentration of 0.5 μg/ml. The cells were radiolabeled for the last 4 or 18 h of culture with 0.25 or 0.5 μCi of ^{125}iododeoxyuridine (^{125}IUDR; ICN Biomedicals, Inc., Costa Mesa, Calif.) and harvested onto glass filters with an automated cell harvester (Skatron Instruments, Inc., Sterling, Va.). Proliferation was determined by measuring the incorporation of ^{125}IUDR by counting the radioactivity on the filters in a gamma counter (Packard, Laguna Hills, Calif.). Results are expressed as the mean radioactivity in counts per minute of triplicate samples ± standard deviation.

RESULTS

Clinical responses of cattle infected and challenged with *B. bovis*. Animal C15, inoculated with autologous, cultured *B. bovis*-infected erythrocytes, experienced mild signs of infection after the primary inoculation, with a 24% reduction in PCV on day 7 postinoculation. There were no clinical signs of babesiosis following the secondary and tertiary inoculations. This cow was not treated with Ganaseg. Animals C88 and C97 developed severe babesiosis following infestation with *B. bovis*-infected ticks. Parasites were observed in blood films from C88 on days 8 to 13 postinfestation, and on day 10 the PCV was reduced by 48%. Parasites were detected in blood films from C97 on days 13 and 14 postinfestation, and on day 13 the PCV fell by 50%. Both animals were treated with Ganaseg. Three months later, C88 and C97 were challenged with an infective tick stabilate, and both animals were solidly immune to challenge. They experienced no reduction in PCV, whereas the nonimmune control animal C110 experienced severe babesiosis with a 57% reduction in PCV on day 14 postinoculation. This animal recovered without treatment.

TABLE 1. Comparison of proliferative responses of normal and immune PBMC cultured for 3 or 6 days with *B. bovis* antigen or mitogen

Cattle	Radioactivity (cpm) incorporated by PBMC with indicated antigen or mitogen[a]		
	URBC CM	*B. bovis* CM	ConA
C99 (control)			
Day 3	382 ± 24	389 ± 30	28,750 ± 4,435
Day 6	431 ± 60	1,290 ± 715	40,306 ± 9,050
C15 (immune)			
Day 3	1,011 ± 164	3,080 ± 294	119,530 ± 5,485
Day 6	3,683 ± 2,956	77,502 ± 8,798	59,202 ± 4,372
C97 (immune)			
Day 3	480 ± 27	961 ± 165	126,858 ± 3,258
Day 6	673 ± 25	16,457 ± 3,990	55,182 ± 2,865

[a] A total of 2×10^5 PBMC were cultured in wells of half-area 96-well plates with antigen or ConA. Background responses (medium) were always less than the responses to URBC CM. After 3 or 6 days, the cells were radiolabeled for 4 h with ^{125}IUDR and then harvested and counted as described in the text. Results are means ± standard deviations of triplicate cultures. ConA was present at a final concentration of 1 μg/ml; URBC and *B. bovis* CM antigens were present at a final concentration of 25 μg/ml.

Serologic responses of *B. bovis*-infected cattle. All four animals were serologically positive for *B. bovis*, as determined by indirect immunofluorescence staining of smears of cultured parasites (not shown). Postchallenge sera from all animals reacted on immunoblots with numerous bands present in whole merozoites and soluble and membrane subfractions of merozoites, whereas there was no reactivity against proteins present in uninfected, cultured erythrocytes. Figure 1 is an immunoblot of preimmune (lanes e to h) and postchallenge (lanes a to d) sera of animals C15 (Fig. 1A) and C97 (Fig. 1B) incubated with URBC and *B. bovis* MER, CM, and HSS. Major protein bands in the MER recognized by C97 antiserum had approximate molecular masses of 42, 60, 85, 120, and 225 kDa (Fig. 1B, lane b), whereas C15 immune serum reacted most strongly with bands of 42 and 116 kDa (Fig. 1A, lane d). Several protein bands recognized by immune sera from C15 and C97 were present in both soluble and membrane fractions, including the immunodominant 42-kDa (12, 15), 60-kDa (12, 40), and 85-kDa (12) proteins. Serum from normal animal C99 did not react with any babesial protein, and none of the sera reacted with *B. bigemina* merozoites on immunoblots (data not shown).

Examination of babesia-specific proliferative responses over time. To determine the optimal assay time for babesia-specific responses, proliferation assays of control (C99) or

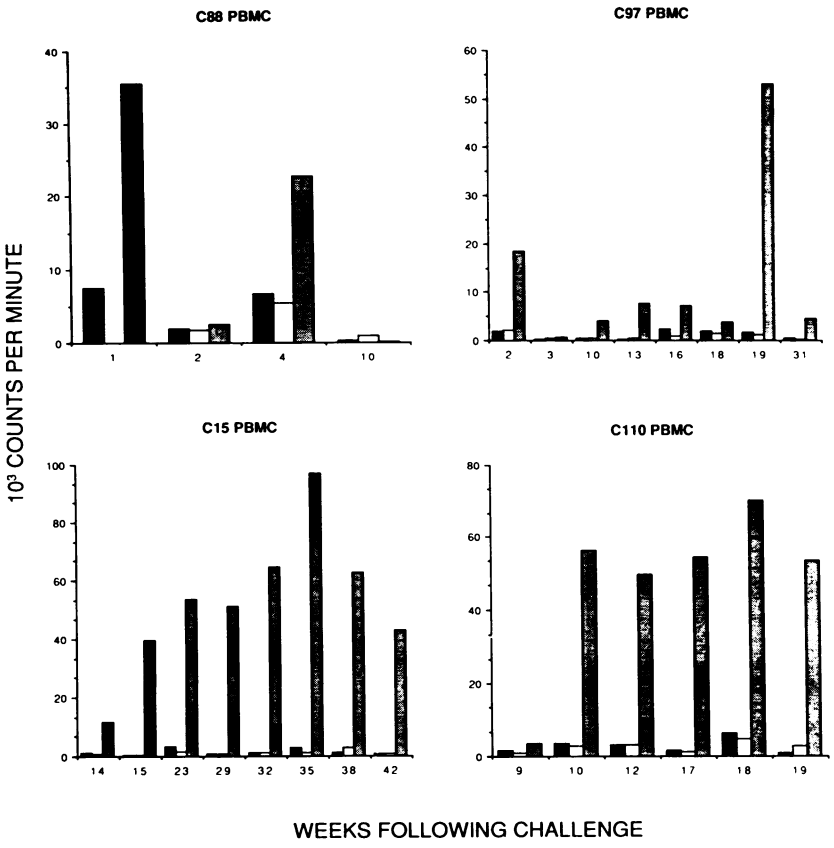

FIG. 2. Time course of the proliferative response of PBMC from *B. bovis*-immune cattle. PBMC (4×10^5) from immune cattle were assessed for proliferative responses against *B. bovis* MER (gray bars), URBC (white bars), or medium control (black bars) at various weeks following the last challenge inoculation as indicated on the abscissa. Cells were cultured for 6 or 7 days with indomethacin, radiolabeled for 4 h, harvested, and counted as described in the text. The data shown are for optimal concentrations of URBC or MER antigen in the culture wells, which were as follows: C88, 6.2 μg/ml; C97, 25 μg/ml; C15, 1.0 to 6.2 μg/ml; and C110, 25 μg/ml.

immune (C97 and C15) PBMC were performed for 3 or 6 days (Table 1). There was no response to URBC at either day. Little or no reactivity against babesial antigen had occurred by day 3, but strong responses were present by day 6. In contrast, strong responses to the T-cell mitogen ConA were present by day 3. Subsequent assays were conducted for 5 to 7 days.

Immune cattle were monitored for proliferative responses to babesial and control antigens for weeks or months following the last challenge inoculation. Figure 2 presents the responses of PBMC from the four immunized cattle to optimal antigen concentrations of purified merozoites or uninfected erythrocytes on various weeks after challenge. Vigorous proliferative responses to unfractionated merozoites were consistently observed with C15 and C110 PBMC after 10 to 15 weeks postchallenge. Animals C88 and C97 had inconsistent and often undetectable responses to merozoites over time. However, responses to the CM fraction were consistently positive in all immune animals and after 2 years could still be induced in PBMC from animals C97 and C15, which were chosen for prolonged study.

Differential responses of PBMC from babesia-sensitized cattle to different antigenic fractions. PBMC from *B. bovis*-immune and control cattle were compared for stimulation by different crude preparations of uninfected erythrocytes and *B. bovis* merozoites. Figure 3 compares proliferation of PBMC from three immune cattle and normal animal C99 against four concentrations of URBC, MER, CM, and HSS fractions. PBMC from all animals were unresponsive to URBC (Fig. 3) and soluble and membrane fractions prepared from URBC (not shown). PBMC from all immune cattle responded vigorously to the parasite CM fraction in a dose-dependent manner, with maximal proliferation occurring at a concentration of 25 μg of protein per ml. Soluble HSS was weakly stimulatory for C97 PBMC and moderately stimulatory for C110 PBMC, but it induced strong responses in C15 PBMC at the higher concentrations of antigen (5 and 25 μg of protein per ml). MER induced intermediate levels of proliferation in C97 and C110 PBMC, in a dose-dependent fashion, whereas the proliferative response of C15 PBMC to MER was routinely highest with the lowest concentration of protein assayed (in this case, 0.2 μg/ml). Occasional responses of control C99 lymphocytes to CM or HSS antigen were observed, but these responses were neither consistent nor dose dependent. Nevertheless, the possibility that merozoites contain mitogenic components must be considered.

Effect of indomethacin on parasite-specific responses. We observed large, activated mononuclear cells in cultures of PBMC stimulated with merozoites. This, together with the finding that high concentrations of merozoite antigen had an inhibitory effect on proliferation of C15 PBMC (Fig. 3), prompted us to test the effect of the prostaglandin E_2 (PGE_2) inhibitor indomethacin on parasite-induced proliferation. At a final concentration of 0.5 μg/ml, indomethacin has been

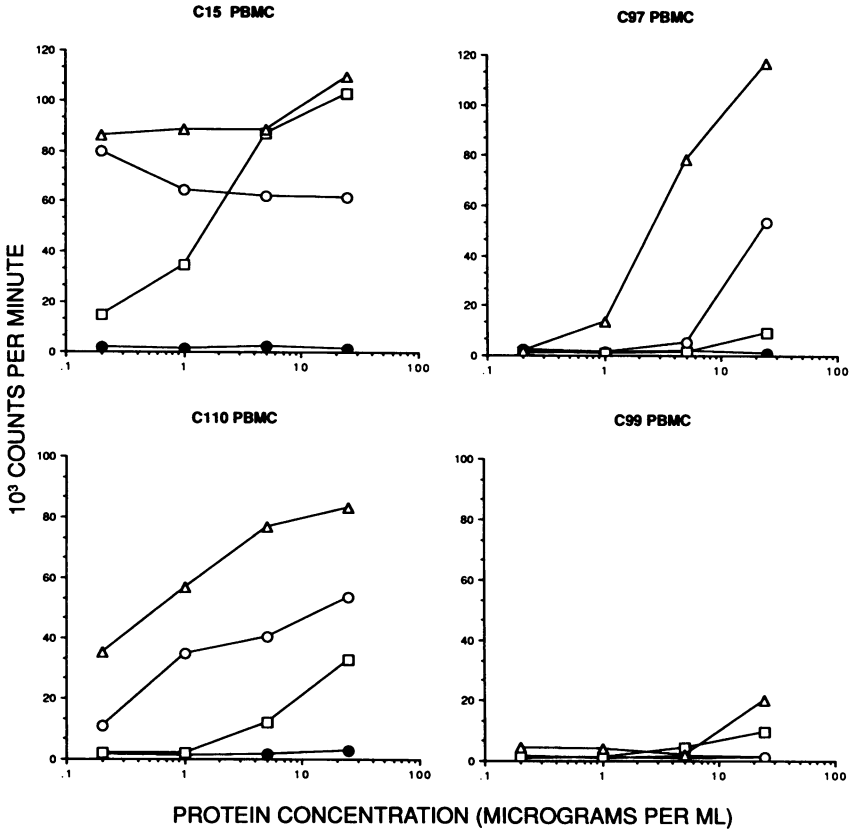

FIG. 3. Dose-dependent proliferative responses against merozoite fractions. PBMC (4×10^5) from one normal (C99) and three immune cattle were stimulated for 6 days with a final concentration of 0.2, 1, 5, or 25 μg of protein per ml of URBC (solid circles), *B. bovis* MER (open circles), *B. bovis* CM (triangles), or *B. bovis* HSS (squares). Indomethacin was included in the assay.

shown to augment interleukin-2 (IL-2) production by bovine T cells (35). When added to proliferation assays, this concentration of indomethacin enhanced the responses of PBMC from all immune cattle to MER and the CM fraction; an eightfold increase was observed in the proliferation of C97 PBMC to CM with indomethacin (Table 2). There was less effect on the response to HSS and no effect on the response to URBC.

Inhibitory effect of merozoites. Because the CM fraction from merozoites induced higher levels of proliferation than intact merozoites, the possibility that merozoites were inhibitory or induced a downregulation of a proliferative response was examined. The effect of MER or URBC antigen on ConA-induced blastogenesis of normal and immune PBMC is shown in Fig. 4. With PBMC from three animals (C99, C97, and C15), there was negligible (5% or less) inhibition of the response to ConA alone when URBC was added with ConA to the assay wells. However, when MER was added with ConA in the assay, inhibition of the ConA response ranged from 46 to 54%. The ConA response of PBMC from the fourth animal (C110) was inhibited 12% by URBC and 34% by MER. The inhibition of the ConA response by MER was evident in 6-day (Fig. 4) but not 3-day assays, and inhibition by either the CM or HSS fraction was not appreciably different from that by URBC (not shown).

Proliferative responses to soluble culture supernatant antigens. Supernatants from cultures containing a high percentage of parasitized erythrocytes or uninfected erythrocytes were compared for antigenic activity at a protein concentra-

TABLE 2. Effect of indomethacin on cell-mediated immune responses

Antigen or mitogen[a]	Radioactivity (cpm) incorporated by PBMC[b]		
	C15	C97	C110
Medium			
Alone	1,004 ± 102	1,084 ± 180	839 ± 22
+ Indo	2,676 ± 167	1,151 ± 99	1,013 ± 150
ConA			
Alone	129,173 ± 1,809	115,148 ± 3,543	41,867 ± 249
+ Indo	145,463 ± 247	126,690 ± 4,119	80,954 ± 3,107
URBC			
Alone	1,117 ± 114	912 ± 72	829 ± 174
+ Indo	2,965 ± 495	1,050 ± 85	980 ± 31
MER			
Alone	52,666 ± 4,475	2,005 ± 210	22,365 ± 3,910
+ Indo	91,004 ± 9,444	4,445 ± 1,582	40,506 ± 10,348
CM			
Alone	87,066 ± 909	6,745 ± 1,450	24,187 ± 334
+ Indo	140,492 ± 2,034	55,564 ± 7,627	52,324 ± 4,948
HSS			
Alone	78,908 ± 2,965	1,669 ± 577	7,476 ± 164
+ Indo	144,425 ± 8,729	2,505 ± 1,268	8,204 ± 590

[a] ConA and antigen were present at a final concentration of 5 μg/ml, and indomethacin (Indo) was included in some wells.

[b] A total of 4×10^5 PBMC from three *B. bovis*-immune cattle were cultured with medium, antigen, or ConA for 5 (C15 and C97) or 7 (C110) days, radiolabeled for 4 h, and harvested as described in the text. Results are means ± standard deviations of triplicate cultures.

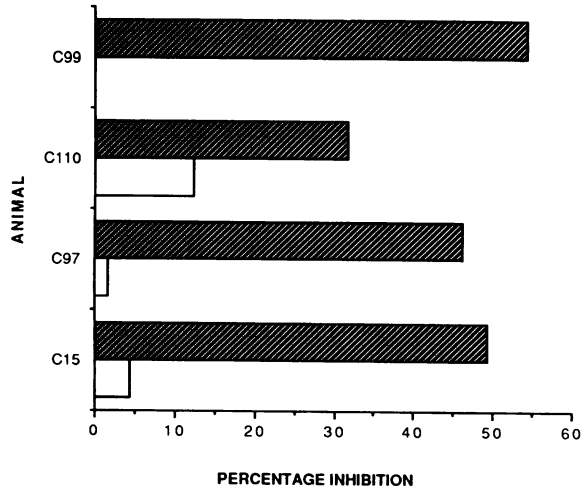

FIG. 4. Inhibition of ConA-induced proliferation by merozoites. PBMC (4×10^5) from one normal (C99) and three immune cattle were stimulated for 6 days with ConA (at a final concentration of 1 μg/ml), MER (at a final concentration of 25 μg/ml), URBC (at a final concentration of 25 μg/ml), or a combination of ConA plus MER or ConA plus URBC. Percent inhibition of the proliferative response to ConA alone is shown for PBMC cultured with ConA plus MER (hatched bars) and ConA plus URBC (white bars). Indomethacin was included in the assay.

tion of 250 μg/ml (Fig. 5). PBMC from animal C15, which was immunized with cultured parasites, were consistently responsive to ISUP and, at a protein concentration of 50 μg/ml, incorporated 36,715 ± 3,403 cpm. In contrast, PBMC from cattle infected with tick-derived parasites (C97 and C110) did not respond to the lower antigen concentration and responded only weakly to the higher concentration of ISUP.

Proliferative responses to unrelated *B. bigemina* merozoites. *B. bigemina* merozoites also induced proliferation of PBMC

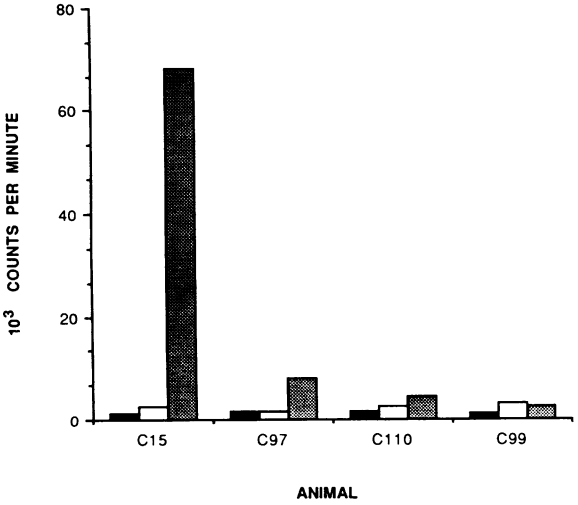

FIG. 5. Proliferative responses to culture supernatant antigen. PBMC from one normal (C99) and three immune cattle were stimulated for 6 days with medium alone (black bars) or with supernatants USUP (white bars) or ISUP (gray bars). The responses shown were elicited with a final protein concentration of 250 μg/ml. Indomethacin was included in the assay.

TABLE 3. Proliferative responses of PBMC to *B. bigemina* merozoites

Antigen and concn (μg/ml)	Radioactivity (cpm) incorporated by PBMC[a]	
	C15	C97
Medium	966 ± 308	647 ± 66
URBC CM		
5	626 ± 83	777 ± 316
25	834 ± 391	535 ± 33
B. bovis CM		
5	20,396 ± 1,422	3,421 ± 1,141
25	22,831 ± 756	8,147 ± 3,596
B. bigemina MER		
5	15,703 ± 1,786	5,018 ± 1,623
25	14,386 ± 1,948	793 ± 262

[a] A total of 2×10^5 PBMC were cultured in half-area 96-well plates with antigen for 6 days, radiolabeled for 4 h with ^{125}IUDR, harvested, and counted as described in the text. All wells contained indomethacin. Results are means ± standard deviations of triplicate cultures.

from animals C15 and C97, although these animals were serologically unreactive with this parasite on immunoblots. The response of C15 PBMC was always vigorous to this parasite, whereas the response of C97 PBMC was routinely weaker (Table 3).

Role of monocytes/macrophages in the response to babesial antigens. Other protozoan parasites, including *Plasmodium falciparum* (45, 49) and *Theileria parva* (1, 11, 30, 31), have been shown to induce nonspecific lymphocyte proliferation. To examine the possibility that babesial parasites were also mitogenic, PBMC depleted of monocytes by 2 h of adherence to polystyrene (10) were compared with unseparated PBMC for the ability to respond to *Babesia* antigen or ConA (Table 4). Adherent cells were required for a babesia-specific response in both immune animals but not for a mitogenic response to ConA. In undepleted PBMC, both specific and nonspecific responses were markedly enhanced by the addition of indomethacin. However, in monocyte-depleted cells, indomethacin had little effect on the ConA response and no effect on the babesia-specific response. These data suggest that monocytes/macrophages are required as antigen-presenting cells for the induction of lymphocyte blastogenesis to babesial antigens.

DISCUSSION

The results of this study demonstrate that infection of cattle with *B. bovis*-infected ticks, tick stabilate, or cultured merozoites elicits strong, cell-mediated immune responses to merozoite antigens. All four immunized animals, but not the control animal, responded to parasite antigens in a dose-dependent manner. Animals C15 and C97 have been monitored for 2 years since the last parasite inoculation, and they continue to respond vigorously to parasite antigen. Since both animals received viable parasites upon challenge, these long-lived responses may be due to chronic infection and continual release of parasite antigen or to a memory T-cell response.

When the proliferative responses against crude, subcellular fractions of merozoites were compared, we found that cattle infected with tick-derived parasites preferentially responded to an antigenic fraction enriched in parasite membranes. In contrast, the animal that was inoculated with cultured merozoites responded vigorously to the membrane-enriched fraction, the soluble merozoite fraction, and solu-

TABLE 4. Requirement of plastic-adherent PBMC for *B. bovis*-specific proliferative responses

Cattle and PBMC	Radioactivity (cpm) incorporated by PBMC with indicated antigen or mitogen[a]		
	Medium	*B. bovis* MER	ConA
C88			
Unseparated			
Alone	2,022 ± 297	4,517 ± 656	51,905 ± 676
+ Indo[b]	7,424 ± 2,954	35,580 ± 7,752	119,952 ± 3,966
Nonadherent[c]			
Alone	793 ± 86	1,026 ± 123	111,483 ± 1,368
+ Indo	716 ± 66	881 ± 117	117,363 ± 3,240
C97			
Unseparated			
Alone	8,875 ± 110	48,749 ± 8,945	62,056 ± 2,364
+ Indo	7,231 ± 958	71,027 ± 7,504	98,306 ± 5,355
Nonadherent			
Alone	438 ± 38	705 ± 123	84,387 ± 5,595
+ Indo	279 ± 197	660 ± 155	91,160 ± 5,270

[a] A total of 4×10^5 PBMC were cultured in 96-well plates with medium, antigen, or ConA for 7 days. The cells were radiolabeled for 18 h with ^{125}IUDR, harvested, and counted as described in the text. Results are means ± standard deviations of triplicate cultures. ConA was present at a final concentration of 5 μg/ml; *B. bovis* MER antigen was present at a final concentration of 6.2 μg/ml.

[b] Indomethacin (Indo) was included in the assay.

[c] A total of 5×10^7 PBMC were incubated for 2 h at 37°C in a 25-cm^2 flask (10 ml), and the nonadherent cells were used in the assay.

ble culture supernatant antigens. Responses of animals C97 and C110 to the soluble merozoite fraction were weaker, even in the presence of indomethacin, and these cattle did not react significantly with culture supernatant exoantigens. These studies suggest that antigens most immunogenic for T cells, and those which induce an anamnestic response from cattle exposed to babesia-infected ticks, are found in parasite membrane-enriched fractions. Soluble antigens and antigens secreted or shed into the culture supernatant most likely contain some of these membrane-associated proteins, as shown by the reactivity on immunoblots of immune sera with protein bands common to CM and HSS fractions. However, the use of soluble parasite extracts to induce T-cell blastogenesis may explain the weak and short-lived responses in *B. bovis*-immune cattle observed by Timms and coworkers (43). Furthermore, the unique response of PBMC from animal C15 to culture supernatant antigens may be directed primarily against cultured merozoite antigens used for immunization. The response of *B. bovis*-immune PBMC to *B. bigemina* parasites is likely directed against epitopes shared by the two parasites, and species-common polypeptides have been demonstrated by immunoprecipitation with immune sera (26). The lack of serological reactivity on immunoblots with C97 and C15 immune sera may reflect a difference in T- and B-cell epitopes on these putative cross-reactive proteins.

The optimal time of 5 to 7 days for blastogenesis to occur in response to babesial antigen is consistent with an antigen-induced and not a mitogen-induced response. Furthermore, comparison of proliferative responses from immune and normal cattle indicates that these responses were evoked in an antigen-specific manner, since babesial antigens stimulated little or no proliferation in PBMC from the nonimmune control. Additional evidence for the antigen-specific nature of the proliferative response was demonstrated by the inability of PBMC depleted of plastic-adherent cells to respond to antigen, with no reduction in the response to ConA. Because the majority of monocytes are removed by this procedure (10), these results indicate that monocytes are required as antigen-presenting cells in the babesia-specific response.

The regulation of lymphocyte proliferation in response to babesial antigens appears quite complex. As well as the apparent requirement for monocytes as accessory or antigen-presenting cells, monocytes appear to inhibit immune responses through the production of PGE_2. This autocoid has been shown to play a key role in immune downregulation by inhibiting both IL-1 and IL-2 production (reviewed in references 4 and 8), possibly through the induction of suppressor T cells (5). In our studies, the PGE_2 inhibitor indomethacin enhanced both babesia-specific and ConA responses but did not reverse the inability of uninfected erythrocyte membranes to stimulate PBMC. An interpretation of these results is that babesial merozoite antigens induce the production of PGE_2 by monocytes, which may downregulate IL-1- and IL-2-induced lymphocyte proliferative responses. Our findings are consistent with those of Ruebush and coworkers (34), who showed that *B. microti* infection of mice elicited PGE_2 synthesis by macrophages, which resulted in suppression of delayed-type hypersensitivity responses to parasite antigens.

Unfractionated merozoites both stimulated proliferation of antigen-primed lymphocytes and inhibited the proliferative response to the T-cell mitogen ConA. This inhibitory effect was observed in the presence or absence of indomethacin, implying that factors in addition to PGE_2 are involved in suppressing proliferation. We are exploring the possibility that tumor necrosis factor alpha, released by parasite-activated macrophages, may play a role in downregulating T-cell proliferation. Inhibition of ConA-induced proliferation was not caused by antigen-specific T cells, since PBMC from nonimmune animal C99 were inhibited to the same extent as those from babesia-sensitized animals. Soluble factors produced by *T. parva*-infected T cells were similarly shown to suppress proliferation of T cells from normal cattle (11).

These studies provide a foundation for further analyses of the cellular immune response which is undoubtedly involved in protective immunity against *Babesia* spp. and all hemoparasites. To unravel the complex interactions between host lymphocytes, monocytes, and parasite antigens, defined subpopulations of T cells and biochemically purified or recombinant parasite antigens must be used. Experiments designed to identify T-cell-immunodominant babesial antigens by using functionally characterized babesia-specific T-cell lines and clones are in progress and will be the subject of future publications.

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REFERENCES

1. **Brown, W. C., and K. S. Logan.** 1986. Bovine T-cell clones infected with *Theileria parva* produce a factor with IL 2-like activity. Parasite Immunol. **8**:189–192.
2. **Brown, W. C., C. Sugimoto, P. A. Conrad, and D. J. Grab.** 1989. Differential response of bovine T-cell lines to membrane and soluble antigens of *Theileria parva* schizont-infected cells. Parasite Immunol. **11**:567–583.
3. **Callow, L. L.** 1977. Vaccination against bovine babesiosis, p.

121–149. *In* L. H. Miller, J. A. Pino, and J. J. McKelvey (ed.), Immunity to blood parasites of animals and man. Plenum Publishing Corp., New York.
4. **Chang, J., and A. J. Lewis.** 1984. Prostaglandins and cyclooxygenase inhibitors, p. 649–667. *In* R. L. Fenichel and M. A. Chirigos (ed.), Immune modulation agents and their mechanisms. Marcel Dekker, New York.
5. **Chouaib, S., L. Chatenoud, D. Klatzmann, and D. Fradelizi.** 1984. The mechanisms of inhibition of human IL 2 production. II. PGE_2 induction of suppressor T lymphocytes. J. Immunol. **132**:1851–1857.
6. **Chulay, J. D.** 1989. Development of sporozoite vaccines for malaria. Trans. R. Soc. Trop. Med. Hyg. **83**(Suppl.):61–66.
7. **Clark, I. A., W. B. Cowden, G. A. Butcher, and N. H. Hunt.** 1987. Possible roles of tumor necrosis factor in the pathology of malaria. Am. J. Pathol. **129**:192–199.
8. **Cueppens, J. L., and J. S. Goodwin.** 1984. Prostaglandins as modulators of T and B lymphocyte function, p. 627–648. *In* R. L. Fenichel and M. A. Chirigos (ed.), Immune modulation agents and their mechanisms. Marcel Dekker, New York.
9. **Emery, D. L.** 1981. Adoptive transfer of immunity to infection with *Theileria parva* (East Coast fever) between cattle twins. Res. Vet. Sci. **30**:364–367.
10. **Goddeeris, B. M., C. L. Baldwin, O. ole-MoiYoi, and W. I. Morrison.** 1986. Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. Functional evaluation of monocytes in response to lectins. J. Immunol. Methods **89**:165–173.
11. **Goddeeris, B. M., and W. I. Morrison.** 1987. The bovine autologous *Theileria* mixed leukocyte reaction: influence of monocytes and phenotype of the parasitized stimulator cell on proliferation and parasite specificity. Immunology **60**:90–96.
12. **Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire.** 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. **56**: 2363–2368.
13. **Goff, W. L., and C. E. Yunker.** 1986. *Babesia bovis*: increased percentage of parasitized erythrocytes in cultures and assessment of growth by incorporation of (^{3}H) hypoxanthine. Exp. Parasitol. **62**:202–210.
14. **Grau, G. E., L. F. Fajardo, P. F. Piguet, B. Allet, P. H. Lambert, and P. Vassali.** 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science **237**: 1210–1212.
15. **Hines, S. A., T. F. McElwain, G. M. Buening, and G. H. Palmer.** 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol. **37**:1–10.
16. **Holman, P. J., K. A. Waldrup, and G. G. Wagner.** 1988. *In vitro* cultivation of a Babesia isolated from a white-tailed deer (*Odocoileus virginianus*). J. Parasitol. **74**:111–115.
17. **Kumar, S., M. F. Good, F. Dontfraid, J. M. Vinetz, and L. H. Miller.** 1989. Interdependence of $CD4^+$ T cells and malarial spleen in immunity to *Plasmodium vinckei vinckei*. J. Immunol. **143**:2017–2023.
18. **Kuttler, K. L., M. G. Levy, M. A. James, and M. Ristic.** 1982. Efficacy of a nonviable culture-derived *Babesia bovis* vaccine. Am. J. Vet. Res. **43**:281–284.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Levy, M. G., and M. Ristic.** 1980. *Babesia bovis*: continuous cultivation in a microaerophilus stationary phase culture. Science **207**:1218–1220.
21. **Lunsden, W. H. R., and G. J. C. Hardy.** 1965. Nomenclature of living parasite material. Nature (London) **205**:1032.
22. **Mahoney, D. F.** 1986. Studies on the protection of cattle against *Babesia bovis* infection, p. 539–554. *In* W. I. Morrison (ed.), The ruminant immune system in health and disease. Cambridge University Press, Cambridge.
23. **Mahoney, D. F., J. D. Kerr, B. V. Goodger, and I. G. Wright.** 1979. The immune response of cattle to *Babesia bovis* (syn. *B. argentina*): studies on the nature and specificity of protection. Int. J. Parasitol. **9**:297–306.
24. **Mahoney, D. F., and I. G. Wright.** 1976. *Babesia argentina*: immunization of cattle with a killed antigen against infection with a heterologous strain. Vet. Parasitol. **2**:273–306.
25. **Mahoney, D. F., I. G. Wright, and B. V. Goodger.** 1981. Bovine babesiosis: the immunization of cattle with fractions of erythrocytes infected with *Babesia bovis* (syn. *B. argentina*). Vet. Immunol. Immunopathol. **2**:145–156.
26. **McElwain, T. F., G. H. Palmer, W. L. Goff, and T. C. McGuire.** 1988. Identification of *Babesia bigemina* and *Babesia bovis* merozoite proteins with isolate- and species-common epitopes recognized by antibodies in bovine immune sera. Infect. Immun. **56**:1658–1660.
27. **Miller, K. L., P. H. Silverman, B. Kullgren, and L. J. Mahlmann.** 1989. Tumor necrosis factor alpha and the anemia associated with murine malaria. Infect. Immun. **57**:1542–1546.
28. **Montenegro-James, S., and M. Ristic.** 1985. Heterologous strain immunity in bovine babesiosis using a culture-derived soluble *Babesia bovis* immunogen. Vet. Parasitol. **18**:321–337.
29. **Morrison, W. I., B. M. Goddeeris, W. C. Brown, C. L. Baldwin, and A. J. Teale.** 1989. *Theileria parva* in cattle: characterization of infected lymphocytes and the immune responses they provoke. Vet. Immunol. Immunopathol. **20**:213–237.
30. **Pearson, T. W., L. B. Lundin, T. T. Dolan, and D. A. Stagg.** 1979. Cell-mediated immunity to *Theileria*-transformed cell lines. Nature (London) **281**:678–680.
31. **Pinder, M., S. Kar, K. S. Withey, L. B. Lundin, and G. E. Roelants.** 1981. Proliferation and lymphocyte stimulatory capacity of *Theileria*-infected lymphoblastoid cells before and after the elimination of intracellular parasites. Immunology **44**:51–60.
32. **Ristic, M., and I. Kakoma.** 1988. Exoantigens of *Babesia*, p. 131–141. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
33. **Ruebush, M. J., and W. L. Hanson.** 1980. Thymus dependence of resistance to infection with *Babesia microti* of human origin in mice. Am. J. Trop. Med. Hyg. **29**:507–515.
34. **Ruebush, M. J., L. K. Steel, and D. A. Kennedy.** 1986. II. Prostaglandin-mediated suppression of delayed-type hypersensitivity to infected erythrocytes during *Babesia microti* infection of mice. Cell. Immunol. **98**:300–310.
35. **Sambhara, S. R., and E. L. Belden.** 1988. Bovine interleukin-2: production and characterization. Vet. Immunol. Immunopathol. **18**:165–172.
36. **Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig.** 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Science **330**:664–667.
37. **Scott, P., P. Caspar, and A. Sher.** 1990. Protection against *Leishmania major* in Balb/c mice by adoptive transfer of a T cell clone recognizing a low molecular weight antigen released by promastigotes. J. Immunol. **144**:1075–1079.
38. **Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher.** 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. **168**:1675–1684.
39. **Smith, R. D., J. Carpenter, A. Cabrera, S. D. Gravely, E. E. Erp, M. Osorno, and M. Ristic.** 1979. Bovine babesiosis: vaccination against tick-borne challenge exposure with culture-derived *Babesia bovis* immunogens. Am. J. Vet. Res. **40**:1678–1682.
40. **Suarez, C. E., G. H. Palmer, D. P. Jasmer, S. A. Hines, L. E. Perryman, and T. F. McElwain.** 1991. Characterization of the gene encoding a 60 kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasitol. **46**:45–52.
41. **Timms, P.** 1989. Development of babesial vaccines. Trans. R. Soc. Trop. Med. Hyg. **83**(Suppl.):73–79.
42. **Timms, P., J. J. Dagliesh, D. N. Barry, C. K. Dimmock, and B. J. Rodwell.** 1973. *Babesia bovis*: comparison of culture-derived parasites, non-living antigen and conventional vaccine in the protection of cattle against heterologous challenge. Aust.

Vet. J. **60**:75–77.
43. **Timms, P., N. P. Stewart, B. J. Rodwell, and D. N. Barry.** 1984. Immune responses of cattle following vaccination with living and non-living *Babesia bovis* antigens. Vet. Parasitol. **16**:243–251.
44. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
45. **Troye-Blomberg, M., H. Perlmann, E. Patarroyo, and P. Perlmann.** 1983. Regulation of the immune response in *Plasmodium falciparum* malaria. II. Antigen specific proliferative responses in vitro. Clin. Exp. Immunol. **53**:345–353.
46. **Winger, C. M., E. U. Canning, and J. D. Culverhouse.** 1987. Induction of protective immunity to *Babesia divergens* in mongolian gerbils, *Meriones unguiculatus*, using culture-derived immunogens. Vet. Parasitol. **26**:43–53.
47. **Wright, I. G., B. V. Goodger, G. D. Buffington, I. A. Clark, F. Parrodi, and D. J. Waltisbuhl.** 1989. Immunopathophysiology of babesial infections. Trans. R. Soc. Trop. Med. Hyg. **83**(Suppl.): 11–13.
48. **Wright, I. G., B. V. Goodger, and I. A. Clark.** 1988. Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections. Parasitol. Today **4**:214–218.
49. **Wyler, D. J., and J. Brown.** 1977. Malaria antigen-specific T-cell responsiveness during infection with *Plasmodium falciparum*. Clin. Exp. Immunol. **29**:401–407.